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INFLUENCE OF EPIDEMIOLOGICAL FACTORS ON THE BIOHERBICIDAL EFFICACY OF A *Xanthomonas campestris* ISOLATE ON COMMON COCKLEBUR (*Xanthium strumarium*)

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ABSTRACT

Greenhouse and controlled-environment studies were conducted to determine the effects of incubation temperature, dew period temperature, dew period duration, plant growth stage, and cell concentration on the bioherbicidal efficacy of a highly virulent isolate (LVA987) of the bacterial pathogen, *Xanthomonas campestris*, against *Xanthium strumarium* (common cocklebur). *X. campestris* infected cocklebur at 20, 25, 30, and 35ºC but the disease achieved at 20ºC was not sufficient to cause high plant mortality. Plant mortality was also significantly lower in plants that were exposed to < 12 h of dew, or at dew temperatures of 15 or 35 ºC. Plants at the 0-4 leaf stage were controlled more efficaciously than older plants and increasing cell concentration from 10^5 to 10^9 cells ml^{-1} resulted in higher mortality and biomass reduction levels. Results indicate that *X. campestris* can infect and kill cocklebur over a wide range of temperature, dew period, and inoculum levels and, therefore has potential as a bioherbicidal agent against common cocklebur.

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1 Introduction

Common cocklebur (*Xanthium strumarium* L.) is a short-day annual weed of the Asteraceae family that is economically important in soybean [*Glycine max* (L.) Merr.] (Rushing & Oliver, 1998; Webster, 2001; Norsworthy, 2003), cotton (*Gossypium hirsutum* L.) (Byrd & Coble, 1991; Webster, 2001) and peanuts (*Arachis hypogaea* L.) (Royal et al., 1997) in the U.S. It occurs throughout the U.S. and Canada (Anonymous, 2013) and is considered one of the most economically troublesome weeds in the world (Holm et al., 1977). Common cocklebur contains compounds that cause poisoning and death in cattle, horses and swine (Burrows & Tyrl, 1989). Heavy infestations of common cocklebur can also reduce yield by 50 to 80% in soybean (Barrentine, 1974; Bloomberg et al., 1982). It is aggressive, capable of growing 1-2 cm/day (Weaver & Lechowicz, 1982) and its vigorous growth habit (within and above the crop canopy) contributes to its weediness (Regnier et al., 1989). It can grow to about 1.5 m tall with thick, rough leaves up to 12 cm long. Seeds are enclosed inside a bur measuring 2 to 4 cm, with many hooked prickles that attach to clothing or animals, aiding in the spread of this weed (Weaver & Lechowicz, 1982).

Herbicide resistance has been reported in common cocklebur. Several biotypes of cocklebur were found resistant to imazaquin {2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-quinolinecarboxylic acid} (Barrentine, 1994; Abbas & Barrentine, 1995) and MSMA (monosodium methylarsonate) (Haighler et al., 1994; Abbas & Barrentine, 1995). Prior to the availability of glyphosate-resistant soybeans, common cocklebur was controlled with bentazon [3-(1-methylethyl)-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide] and acetolactate synthase (ALS) -inhibiting herbicides (Muyonga et al., 1996). But, resistant biotypes to most ALS-inhibiting herbicides are now reported throughout the north-central and southern U.S. (Heap, 2013). Glyphosate can control common cocklebur (Wiesbrook et al., 2001), but early removal is crucial to avoid yield reduction that can occur as early as 4 wks after planting (Barrentine, 1974). The development of herbicide resistance and the trend towards a more chemically-free environment has increased interest in biological weed control using bioherbicides (Hoagland, 1990; Charudattan, 1991; Charudattan, 2005; Weaver et al., 2007).

There are several reports of disease organisms and/or microbial biocontrol agents of *Xanthium* spp. Over a dozen fungal species infect *Xanthium* spp. in the U.S. and Canada (Weaver & Lechowicz, 1982). The obligate parasitic rust *Puccinia xanthii* Schw., that occurs throughout the U.S., southern Canada, parts of Europe, and India infects several species of *Xanthium* and *Ambrosia* (Conners, 1967; Hasan, 1974; Alcorn, 1975; Jadhav & Somani, 1978). The fungus *Colletotrichum orbiculare* causes anthracnose on stems and leaves of *X. spinosum* and under optimal conditions kills plants in 14 days (Auld et al., 1990). *Alternaria helianthi* (Hansf.) Tubaki and Nishih. also has been evaluated as a bioherbicide for *X. strumarium* (Quimby, 1989; Abbas & Barrentine, 1995; Abbas & Egley, 1996; Sanyal et al., 2008). This fungus, isolated from sunflower (*Helianthus annuus* L.) (Quimby, 1989), also infects other Compositae plants (Allen et al., 1983). A powdery mildew that infects cocklebur in India has been described that might have biological control potential (Sharma, 1981). Other pathogens have been evaluated for controlling *X. strumarium* in India (Deshpande, 1982) and phytotoxins from several fungi and bacteria were found to induce wilt in *X. strumarium* (Kalidas, 1981).

Bacteria were first implicated (ca. 130 years ago) as causal agents in plant disease (Vidaver & Lambrecht, 2004), when a bacterium associated with fireblight of apples and pears was confirmed (Burrill, 1878). Although plant pathogenic bacteria cause numerous diseases of plants throughout the world, the number of diseases, their damage, and economic costs are relatively lower than that caused by fungi or viruses (Kennedy & Alcorn, 1980).

The virulence and host range of a bacterial pathogen, *Xanthomonas campestris* isolate LVA987 as a bio-herbicide against common cocklebur was reported (Boyette & Hoagland, 2013). The present study, was undertaken to determine the effects of various media and temperatures on *in vitro* growth of this bacterium, and the effects of dew period, dew temperature, inoculum concentration and plant growth stages on the biocontrol efficacy of this bioherbicide. Knowledge of these epidemiological parameters is essential for evaluating the bioherbicide potential of *Xanthomonas campestris* for weed control (TeBeest, 1991).

2. Materials and Methods

2.1. Seed Sources and Test Plant Propagation

Common cocklebur seeds were purchased from Azlin Seed Co., (Leland, MS). The burs were soaked in water for 7 days, then planted in a 2:1 mix of jiffy mix:sandy soil (Jiffy Mix, Jiffy Products of America, Inc., Batavia, IL) contained in plastic trays (25 x 52 cm). Germinated seeds were transplanted into 10 cm^2 plastic pots and grown in a greenhouse. Greenhouse conditions were: 28 to 32° C, 40-60% relative humidity (RH), ~14 h day length and 1,650 $\mu E/m^2/s$ photosynthetically active radiation (PAR) measured at midday.

2.2. Effect of incubation temperature and growth medium

A stock culture of *X. campestris* was streaked on Bacto™ nutrient agar (Becton, Dickinson and Co., Sparks, MD) plates and a single colony (isolate LVA987) from this plate was used as inoculum for 100 ml of nutrient broth (Bacto™) in a 250-ml baffled Erlenmeyer flask. The liquid culture was grown overnight at 30°C and 300 rpm in a rotary shaker incubator. This whole culture was mixed 1:1 (v/v) with a sterile 20%

glycerol solution and 2.0 ml aliquots were stored in cryo-vials at -80°C for use as stock cultures. Weekly nutrient agar plates were streaked with stock cultures to produce inocula for growth experiments. *X. campestris* cells in the log phase were used to inoculate flasks for growth studies. Bacto™ liquid growth media were utilized in these studies(i.e., nutrient broth, yeast extract broth, tryptic soy broth, and casamino acids). Distilled water was used as a control.

Log phase cells were obtained by inoculating complex or basal media with cells obtained from streaked nutrient agar plates, followed by growth of these cultures for 12-16 h in a rotary shaker incubator at 30°C and 300 rpm. Prior to use, the generation time for the *X. campestris* cultures was determined by measuring optical density at 1-h intervals. Only cells in the log phase (generation times 150-200 min) were used as inoculum. Cell inocula were prepared by centrifuging whole cultures at 8000 x g for 10 min at 23ºC, decanting the supernatant, and re-suspending the cell pellet in sterile potassium phosphate buffer (12 mM, pH = 6.8). Cell suspensions with an optical density of 1.5 at 620 ηm were used as a 10% inoculum in growth studies, providing an initial cell concentration of 1.0×10^5 cells/ml in the growth medium.

2.3. Effect of air and dew temperature

Five to seven-day old seedlings (cotyledonary to early first-leaf growth stage) of common cocklebur were sprayed until leaves were fully wetted (ca. 100 L/ha) with a formulation of a non-ionic, organosilicone surfactant (Silwet-L77™; OSi Specialties, Inc., Danbury, CT), *Xanthomonas* cells and distilled water (Boyette & Hoagland, 2013).

Final concentration in the formulation was 1.0×10^8 bacterial cells/ml in 0.20% Silwet (v/v). Control plants were sprayed with 0.20% Silwet in distilled water. In this test, plants were placed in individual darkened dew chambers (100% RH) at temperatures of 15, 20, 25, 30, or 35ºC for 16 h. The plants were then transferred to individual growth chambers (Conviron, Model E-7, Pembina, ND) with day/night air temperatures of either 20ºC/10ºC, 25ºC/15ºC, 30ºC/15ºC, 35ºC/25ºC or 40ºC/30ºC. Photoperiods were 14 h at 65% RH, and 820 to 840 $\mu E/m^2/s$ (PAR). Plants were watered daily. Mortality and dry weight reductions were recorded 14 days after treatment.

Dry weight measurements were determined in untreated and treated plants excised at soil level after tissue was dried in an oven (85ºC, 48 h). The experiment was conducted twice with three replications consisting of 12 plants per replicate.

2.4. Effect of dew duration

Common cocklebur seedlings (cotyledonary to early first leaf growth stage) were sprayed until leaves were fully wetted with a spray mix containing 1.0×10^8 bacterial cells/ml in water and Silwet. Control plants were sprayed with 0.20% Silwet in distilled water. Inoculated plants were then placed in darkened dew chambers at 25ºC and 100% RH for periods of 0, 4, 8, 12, 16, 20, or 24 h. Following the dew treatments, plants were placed on sub-irrigated trays on greenhouse benches, and monitored for disease development. Mortality and dry weight reductions were recorded 14 days after treatment. The experiment was conducted twice with three replications consisting of 12 plants per replicate.

2.5. Effect of inoculum concentration and plant growth stage

Common cocklebur plants in the cotyledonary to 1, 2 to 4, 5 to 8, and 9 to 12 leaf stages of growth were sprayed with several concentrations of bacterial cell suspensions, ranging from 1.0×10^5 to 1.0×10^9 cells/ml and held in a dew chamber for 16 h at 25º C.

Control plants were sprayed with 0.20% Silwet in distilled water. After dew treatments, plants were moved to the greenhouse, and mortality and dry weight reductions were recorded 14 days after treatment. Experiments were conducted twice with 3 replications of 12 plants per replicate.

2.6. Experimental design and statistics

Experiments were conducted twice with 3 sets of 12 plants for each experiment. Treatments (in triplicate) were arranged in a randomized complete block design and all experiments were repeated in time. Means were subjected to analysis of variance and were compared with Fisher's LSD (P=0.05) or S.E.M. analysis. All data were analyzed using SAS (Version 9.1, SAS Institute, Inc., Cary, NC) statistical software.

3. Results and Discussion

3.1 Effects of incubation temperature and growth medium

X. campestris cell growth was most rapid at temperatures of 25-30ºC in all of the media that were evaluated (Table 1). Growth was significantly less at low or high temperatures (15º and 40º C). Because there were no significant differences in cell production in nutrient broth, yeast extract broth, or tryptic soy broth (Table 1), nutrient broth was selected for production of inoculate in all subsequent testing.

3.2 Effects of dew and air temperatures

An optimal day, night, and dew temperature regime of 30ºC/25ºC/20ºC was determined for maximum weed mortality (95 %) (Figure 1A) and dry weight reduction (98 %) (Figure 1B) of plants in the cotyledonary to first leaf growth stage. Both weed mortality and biomass reduction were significantly reduced when the day/dew/night temperatures were lowered to 20ºC/15ºC/10ºC. No pathogenesis or mortality occurred on common cocklebur seedlings under a day/dew/night temperature regime of 40ºC/35ºC/30ºC (Figure 1A and 1B).

Table 1 Effect of growth media and temperatures on production of *Xanthomonas campestris*.

Growth medium	Incubation temperature (°C)[1]					
	15	20	25	30	35	40
	Yield (x 10^{10} CFU ml^{-1})					
Nutrient broth	.009a[2]	.08a	1.2a	.89a	.11b	.005a
Yeast extract broth	.008a	1.1a	1.6a	.90a	1.2a	.003a
Tryptic soy broth	.007a	.09a	1.5a	.85a	.10b	.001a
Casamino acids	.001b	.03b	.50b	.38b	.09b	.001a
Distilled H_2O	NG[3]	NG	NG	NG	NG	NG

[1] Generation time = 18 h.

[2] Mean values within columns followed by the same letter do not differ significantly using Fisher's protected least significant difference (P = 0.05).

[3] NG = No growth.

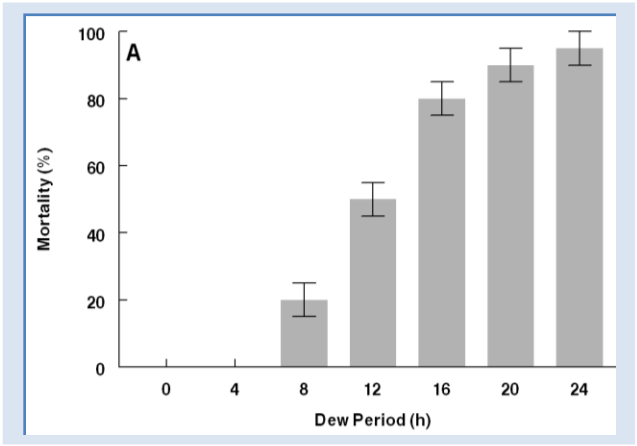

Figure 1A

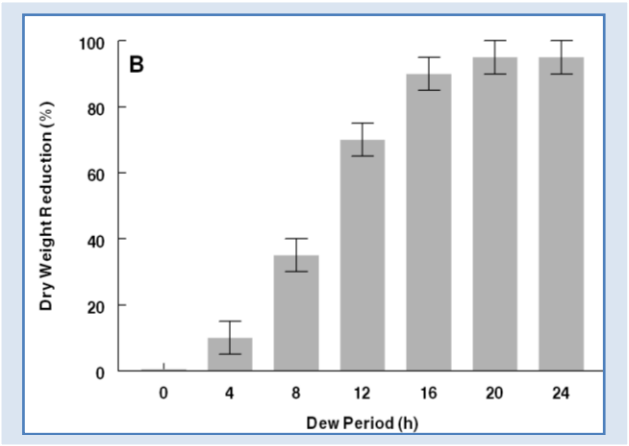

Figure 1B

Figure 1 Effects of dew duration on *X. campestris* (LVA987) efficacy on cocklebur control.
A = mortality; B = dry weight reduction. Dew duration periods ranged from 0-24 h. Error bars represent the LSD value at P = 0.05.

3.2 Effects of dew and air temperatures

An optimal day, night, and dew temperature regime of 30ºC/25ºC/20ºC was determined for maximum weed mortality (95 %) (Figure 1A) and dry weight reduction (98 %) (Figure 1B) of plants in the cotyledonary to first leaf growth stage. Both weed mortality and biomass reduction were significantly reduced when the day/dew/night temperatures were lowered to 20ºC/15ºC/10ºC. No pathogenesis or mortality occurred on common cocklebur seedlings under a day/dew/night temperature regime of 40ºC/35ºC/30ºC (Figure 1A and 1B).

3.3 Effects of dew period duration

The bacterium killed common cocklebur seedlings over a range of dew period durations from 8 - 24 h conducted at 25º C (Figure 2A). A dew period of at least 16 h was required to cause about 80% mortality of plants (Figure 2A). Although no mortality of plants occurred after 4 h of dew, significant dry weight reductions were noted (Figure 2B). Although 100% mortality was not achieved at any dew period (Figure 2A), plants were severely stunted ≥ 50% after 8 - 24 h (data not shown), which resulted in greatly reduced biomass (Figure 2B). Biomass reductions were directly proportional to inoculum concentration applied.

3.4 Effects of plant growth stage and inoculum concentration

Mortality and dry weight reductions under greenhouse conditions were significantly increased at all growth stages by increasing the inoculum concentration (Figure 3A and 3B). Common cocklebur seedlings in the 5 to 8 and 9 to 12 leaf stages were more resistant to infection than younger plants. Weed mortality was significantly less than that achieved with plants at earlier growth stages at the inoculum concentrations tested (Figure 3A). Similar results occurred regarding dry weight reductions of plants at these growth stages and cell concentrations (Figure 3B).

Bacteria belonging to the genus *Xanthomonas* are collectively responsible for diseases on > 400 different host plants, which include many economically important crops (Hayward, 1993). There *Xanthomonas* is a diverse genus containing many pathovars (Dye et al., 1980). These plant-associated bacteria are not known to colonize other environments such as soil or water.

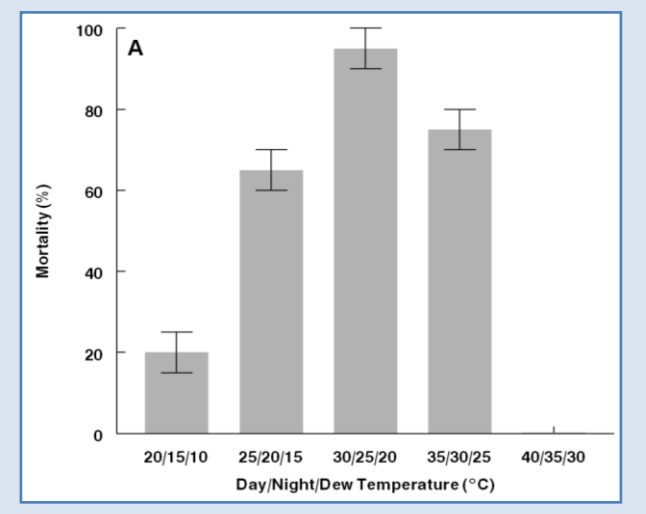

Figure 2A

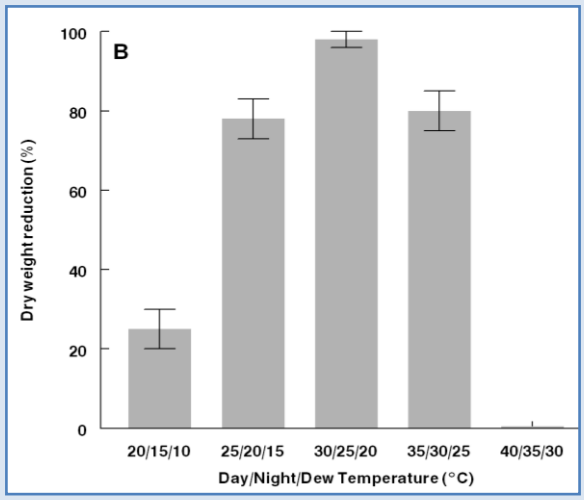

Figure 2B

Figure 2 Effects of day/night/dew temperatures after exposure to dew (16 h) on *X. campestris* (LVA987) efficacy on cocklebur control.
A = mortality; B = dry weight reduction. Error bars represent the LSD value at P = 0.05.

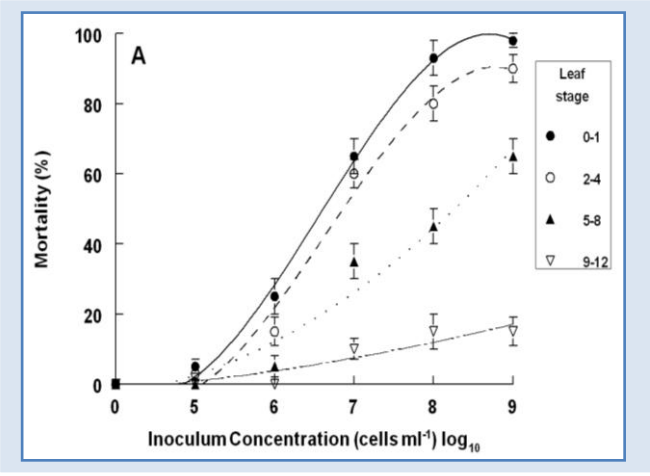

Figure 3A

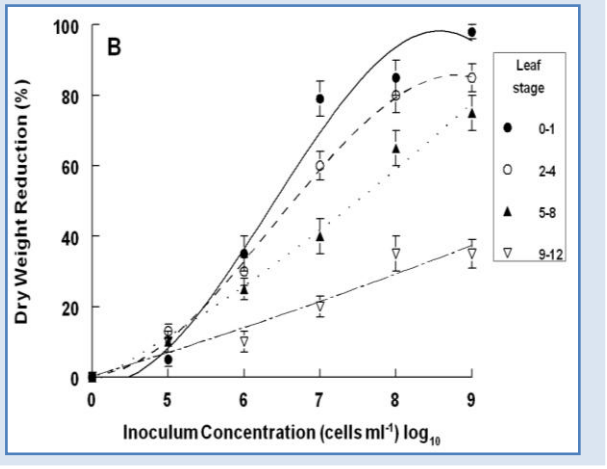

Figure 3B

Figure 3 Effects of *X. campestris* (LVA987) inoculum concentration on common cocklebur control.
A = mortality; B = dry weight reduction. Regression equations for mortality (Solid circles = third degree polynomial, where $Y = 40.33 - 65.26 X + 28.38 X^2 - 2.65 X^3$; $R^2 = 0.99$. Open circles = third degree polynomial, where $Y = 42.67 - 66.59 X + 27.35 X^2 - 2.49 X^3$; $R^2 = 0.98$. Solid triangles = second degree polynomial, where $Y = -4.00 - 1.00 X + 2.14 X^2$; $R^2 = 0.96$. Open inverted triangles = second degree polynomial, where $Y = -3.00 + 1.21 X + 0.36 X^2$; $R^2 = 0.88$). Regression equations for dry weight reduction (Solid circles: = third degree polynomial, where $Y = 21.00 - 43.09 X + 22.92 X^2 - 2.28 X^3$; $R^2 = 0.98$. Open circles = third degree polynomial, where $Y = 12.67 - 25.89 X + 15.44 X^2 - 2.28 X^3$; $R^2 = 0.97$. Solid triangles = second degree polynomial, where $Y = -13.00 + 10.86 X + 0.71 X^2$; $R^2 = 0.96$. Open inverted triangles = second degree polynomial, where $Y = -6.00 + 6.17 X + 0.18 X^2$; $R^2 = 0.94$.
Error bars represent ± 1 S.E.M.

Symptomatic cultivated hosts are generally the best known hosts, whereas weeds and asymptomatic hosts remain difficult to characterize (Mhedbi-Hajri et al., 2013). *Xanthomonas* spp. are used to produce xanthan, a stabilizer and thickener with many applications in the food industry (Gumus et al., 2010).

Although most microbes studied as bioherbicides are fungi, very few bacterial phytopathogens have been examined (Gurusiddaiah et al., 1994; Gealy et al., 1996; Johnson et al., 1996; Caldwell et al., 2012). Several *Xanthomonas* pathovars have also been evaluated as bioherbicides. *X. badrii* was reported as a pathogen of cocklebur in India (Patel et al., 1950). Several pathovars of *X. campestris* have been identified on a wide range of plants including crops and weeds (Anonymous, 1970). *X. campestris* pv. *poae* effectively controlled annual bluegrass (*Poa annua* L.) (Imaizumi et al., 1997), and control was directly proportional to the bacterial concentration applied (Imaizumi et al., 1998*)*. A stable bacterial cell formulation of this organism was developed (Jackson et al., 1998), eventually resulting in a commercial bioherbicidal product (Camperico®) (Nishino & Tateno, 2000). However, successful control of the target weed by this product required wounding (via mowing) of the bluegrass weeds prior to application.

The host specificity of plant pathogenic bacterial strains is generally high and well-characterized, and numerous pathovars have been defined within many species. Plant pathogenic bacteria can occur on non-host plant surfaces without infecting or inciting disease symptomatology. For example, the causal agent of bacterial leaf spot of lettuce (*Lactuca* spp.), *X. campestris* pv. *vitians*, colonized plant surfaces of several weed species in the Asteraceae family, as well as some in the Chenopodiaceae, Malvaceae, Polygonaceae and Portulacaceae families (Toussaint et al., 2012). The number of bacteria on plant surfaces varied significantly among these different weed species and significantly more organisms were recovered on lettuce than on plants in the Chenopodiaceae, Polygonaceae and Portulacaceae families. Although these latter families were not proven as 'true' hosts of *X. campestris* pv. *vitians*, they may play a role in the epidemiology by harboring the pathogen, thus providing a primary inoculum reservoir for infection of true hosts (Toussaint et al., 2012). In our studies with *X. campestris* isolate LVA987, lettuce was also affected, along with several other members of the Asteraceae (Boyette & Hoagland, 2013).

In the previous study, Asteraceae species including marigold, zinnia, sunflower, lettuce and several weeds exhibited varying degrees of injury when challenged with *X. campestris* isolate LVA987. Because of this susceptibility, these non-target plants could become infected if contacted by drift or other off-target dispersal of inoculum from field application of this pathogen used as a bioherbicide. Thus, as with field-scale application of herbicides and other compounds that alter plant growth, biological control agents should be applied using proper safeguards to protect non-target species. In earlier studies we did not examine any of the plants in our host range experiments for bacterial colonization (Boyette & Hoagland, 2013), but this could be the subject of future investigations.

A major constraint of *X. campestris* isolate LVA987, as well as most microorganisms that have been evaluated as bioherbicides, is the requirement for a lengthy period of free moisture (dew) following inoculation. At least 16 h of dew was required to achieve acceptable levels of weed control with this pathogen. Dew periods of this length or adequate free moisture may not always occur in crops where this weed is a problem. However, proper timing of application to weeds in the most susceptible growth stages (cotyledonary to first-leaf stage) would optimize the chances for successful control. Water-in-oil formulations have shown promise in reducing the dew period requirements of some mycoherbicidal fungi (Boyette et al., 1996; Boyette et al., 2010; Boyette et al., 2011) and could possibly be used with this pathogen.

Conclusion

These studies demonstrate that *Xanthomonas* strain LVA987 is effective in controlling common cocklebur over a wide range of physical and environmental conditions. Studies are currently in progress to evaluate the efficacy of this pathogen combined with herbicides such as glyphosate on resistant weeds such as *Conyza* and *Ambrosia* species. These current findings, coupled with work in progress and future studies as outlined above will provide essential knowledge necessary to further evaluate this plant pathogen as a bioherbicidal control of cocklebur and other important weeds.

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